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MEMBRANE-ASSOCIATED REACTIONS IN UBIQUINONE BIOSYNTHESIS IN *ESCHERICHIA COLI*

3-OCTAPRENYL-4-HYDROXYBENZOATE CARBOXY-LYASE

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SUMMARY

A sensitive and quantitative assay for 3-octaprenyl-4-hydroxybenzoate carboxy-lyase has been developed. This enzyme, which catalyses the third reaction in ubiquinone biosynthesis in *Escherichia coli*, was partially purified and some of its properties determined. It was found that a considerable proportion of the carboxy-lyase activity could be separated from the membrane fraction in cell extracts prepared using a French press. Gel filtration showed the molecular weight of the enzyme to be about 340 000. For optimal activity the carboxy-lyase was shown to require Mn^{2+}, washed membranes or an extract of phospholipids, and an unidentified heat stable factor of molecular weight less than 10 000. The carboxy-lyase reaction was also shown to be strongly stimulated by dithiothreitol and methanol. The properties of the carboxy-lyase are compared with the three other enzymes concerned with ubiquinone biosynthesis in *E. coli* which have been studied in vitro. The fact that the substrate of the carboxy-lyase is membrane-bound and the enzyme is stimulated by phospholipid suggests that it normally functions in association with the cytoplasmic membrane in vivo.

INTRODUCTION

Studies with various mutant strains of *Escherichia coli* have elucidated the pathway of biosynthesis of ubiquinone in this organism [1]. The pathway in *E. coli* is similar to a general pathway proposed as a result of work with *Rhodospirillum rubrum* [2]. The isolation of *E. coli* mutants blocked in the various reactions of ubiquinone biosynthesis has allowed the purification of the natural intermediates and has greatly facilitated the study of the enzymes of this membrane-associated pathway in vitro. Apart from chorismate lyase, which is a soluble enzyme catalysing the first specific reaction in ubiquinone biosynthesis [3], the following enzymic activities have been demonstrated in vitro: 4-hydroxybenzoate-octaprenyltransferase [4, 5], 2-octaprenyl-3-methyl-5-hydroxy-6-methoxy-1,4-benzoquinone methyltransferase [6] and 3-octaprenyl-4-hydroxybenzoate carboxy-lyase.

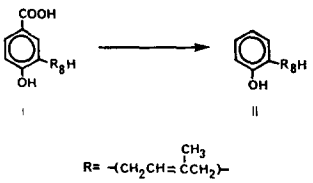


Fig. 1. The reaction catalysed by 3-octaprenyl-4-hydroxybenzoate carboxy-lyase. I, 3-octaprenyl-4-hydroxybenzoate; II, 2-octaprenylphenol.

The first demonstration of the carboxy-lyase reaction (Fig. 1) in vitro was shown by incubating cell extract of *E. coli* with 3-octaprenyl-4-hydroxybenzoate and demonstrating the conversion of the latter to 2-octaprenylphenol by chromatography on silica gel [7]. The activity could not be similarly demonstrated in a *ubiD*$^-$ mutant which accumulated 3-octaprenyl-4-hydroxybenzoic acid even though that mutant formed about 20 % of the normal amount of ubiquinone. Evidence has also been obtained by other workers for the presence of carboxy-lyase activity in smashed cell preparations in *E. coli* [5] and *Rhodospirillum rubrum* [8].

In the present work, a quantitative assay for the direct measurement of 3-octaprenyl-4-hydroxybenzoate carboxy-lyase which does not involve chromatography, has been developed. This assay has been used to study the location and properties of the carboxy-lyase.

MATERIALS AND METHODS

Chemicals

4-Hydroxy-[U-^{14}C]benzoate was prepared by alkali fusion of L-[U-^{14}C]-tyrosine by the method of Parson and Rudney [9]. Unlabelled 3-octaprenyl-4-hydroxybenzoic acid was isolated from strain AN195 as described previously [7]. Biogel A-5M 200–400 mesh was obtained from Bio-Rad Laboratories, Richmond, Calif., U.S.A.

3-Octaprenyl-4-hydroxy-[U-^{14}C]benzoate was prepared as follows. 4-Hydroxy-[U-^{14}C]benzoate (28 nmol, 400 Ci/mol) in methanol was added to a 125-ml flask fitted with a side arm so that the turbidity of the culture could be read in a Klett colorimeter. The methanol was removed by evaporation and after adding water (1 ml) the flask was autoclaved at 120 °C for 15 min. Sterile glucose mineral salts medium (50 ml) with the appropriate supplements for strain AN291 (*ubiD*$^-$, *aroB*$^-$, *metB*$^-$) was added to the flask and the medium inoculated with strain AN291. The culture was grown at 37 °C with shaking until it reached a turbidity of 190 Klett units at which point the concentration of 3-octaprenyl-4-hydroxybenzoate had reached a maximum. The culture was centrifuged and acetone (20 ml) and HCl (5 M, 0.2 ml) were added to the packed cells. The cells were extracted at 60 °C for 3 min, centrifuged and the pellet re-extracted three times with 10 ml of acetone. The acetone extracts were combined, the acetone removed under reduced pressure and, after the addition of 2 ml of water, extracted four times with 4 ml of pentane. The combined pentane extract was concentrated and applied to two Merck Analytical F_{254} silica gel plates (5 × 20 cm) and the plates developed with methanol/chloroform (1 : 9, v/v). Two radioactive bands were found when the plates were scanned, one being ubiquinone

(R_F 0.66) and the other 3-octaprenyl-4-hydroxybenzoic acid (R_F 0.38) which was eluted from the silica gel with methanol (3 × 10 ml). The yield of 3-octaprenyl-4-hydroxy-[U-^{14}C]benzoic acid was 45%. Its identity was confirmed by co-chromatography on silica gel with an unlabelled sample of 3-octaprenyl-4-hydroxybenzoate using the following solvent systems. Methanol : chloroform (1 : 9, v/v), R_F 0.38; ethyl acetate : light petroleum (b.p. 60–80 °C), (1 : 1, v/v), R_F 0.35; and ethyl acetate, R_F 0.50.

Bacterial strains

All of the strains used were derived from *E. coli* K12. They are strains AN164 (*metB*$^-$, *aroB351*), AN256 (*thr-1*, *leu-6*), AN291 (*ubiD410*, *metB*$^-$, *aroB351*). The *aroB*$^-$ allele was introduced into strains as described previously [4].

Media

The glucose-mineral salts medium used and the concentrations of any supplements have been described previously [10]. Media used for growing strains carrying an *aroB*$^-$ allele were supplemented with phenylalanine, 0.2 mM; tyrosine, 0.2 mM; tryptophan, 0.2 mM; 4-aminobenzoate, 1 μM and 2,3-dihydroxybenzoate, 10 μM.

Determination of radioactivity

Radioactivity was determined using a Packard Tri-Carb liquid scintillation spectrometer. The scintillation fluid used consisted of 6 g 2,5-diphenyloxazole and 20 ml ethanol per l of toluene except for aqueous solutions, when Bray's fluid [11] was used.

Assay of 3-octaprenyl-4-hydroxybenzoate carboxy-lyase

The incubation mixture consisted of enzyme preparation (0.2 ml) sodium phosphate buffer (0.05 M, pH 7.0), and additional co-factors as stated, in a total volume of 0.45 ml. Reaction was started by the addition of 3-octaprenyl-4-hydroxy-[U-^{14}C]benzoate (5000 cpm, 10 nmol) in methanol (50 μl). Tests were incubated at 37 °C for 1 h, after which the reaction was stopped by the addition of 2 ml of 0.1 M acetic acid in methanol. The mixture was then extracted three times with 2 ml of pentane after which the combined pentane extract was extracted with 0.1 M potassium hydroxide in 90% methanol (4 ml). After transferring the pentane to a scintillation vial the alkaline methanol was extracted again with 5 ml pentane and the pentane layer added to the scintillation vial. The pentane was completely evaporated, scintillant (10 ml) added to the scintillation vial and the radioactivity determined.

Growth of cells

Cultures were grown either in 2-l flasks containing 1 l of culture shaken at 37 °C or as 10-l cultures in glass fermentor vessels with aeration and stirring. Inocula were grown on plates of nutrient agar containing glucose (30 mM) and, in the case of strains carrying the *aroB351* allele, supplemented with shikimate (0.2 mM).

Preparation of cell extracts

Cells were grown into late logarithmic phase in supplemented glucose-minimal medium, harvested, washed and disrupted in a Sorvall Ribi Cell Fractionator as described previously [6].

Ammonium sulphate fractionation

Nucleic acids were removed from cell extracts by the addition of 70 % of the volume of 2 % protamine sulphate solution needed to completely precipitate the nucleic acids as described previously [6]. Finely powdered ammonium sulphate, 31 g/100 ml (50 % saturation at room temperature) was added slowly with stirring to the protamine sulphate-treated cell extract held at 0 °C. After stirring for a further 30 min, the suspension was centrifuged (15 000 × *g*, 30 min) the supernatant discarded and the pellet dissolved in 0.05 M sodium phosphate buffer (pH 7.0), and diluted to give the original volume before ammonium sulphate treatment. For column chromatography the dissolved pellet was diluted to one fifth of the original volume.

Estimation of protein

Protein was estimated by the method of Lowry et al. [12] using bovine serum albumin as standard.

RESULTS

Development of an assay for 3-octaprenyl-4-hydroxybenzoate carboxy-lyase

To study the carboxy-lyase reaction it was desirable to develop a sensitive and quantitative assay. Both the substrate and product are soluble in non-polar solvents and the obvious difference between them is the lack of an ionisable carboxyl group in the latter (see Fig. 1). Accordingly, an assay was developed based on the method described by Kröger and Klingenberg [13] for the extraction of ubiquinone from mitochondria into light petroleum. Both substrate and product were thus extracted into pentane and the substrate subsequently removed by an extraction under alkaline conditions (see Methods). It was found that extraction of a solution of substrate and product in pentane with 0.1 M KOH in methanol : water (9 : 1, v/v) quantitatively removed the 3-octaprenyl-4-hydroxybenzoic acid from the pentane phase while causing no detectable loss of 2-octaprenylphenol as measured by fluorescence spectroscopy (results not shown).

The smallest amount of 2-octaprenylphenol detectable, using the assay as described in Methods, was about 10 pmol.

Preliminary studies on unfractionated cell extracts

Experiments with unfractionated cell extracts showed that either Tris · HCl or sodium phosphate buffers could be used in the assay of the carboxy-lyase and that, in phosphate buffer, the maximum enzymic activity was achieved with 0.05–0.1 M sodium phosphate buffer (pH 7.0). The lower concentration was used throughout.

As the substrate, 3-octaprenyl-4-hydroxybenzoic acid, is insoluble in water it was added to the incubation mixture in the initial experiments as a methanolic solution. Examination of the effect of methanol on the reaction showed that it stimulated carboxy-lyase activity, the optimal final concentration of methanol being approximately 15 %. The amount of 2-octaprenylphenol formed was increased about 4-fold when the final methanol concentration in the reaction mixture was increased from 2.5 to 15 % (v/v). Of the other organic solvents tested only ethanol gave comparable stimulation of the reaction.

Examination of the time course of the carboxy-lyase reaction showed that

there was a slow decrease in rate with time and an incubation period of 60 min was chosen as the standard incubation time for assays. The amount of product formed was approximately proportional to the concentration of cell extract in the incubation mixture over the range of 2–10 mg of protein added.

Sedimentation properties of the carboxy-lyase

As at least one of the enzymes involved in ubiquinone biosynthesis is membrane-bound, as are intermediates possessing the octaprenyl side chain, the sedimentation properties of the carboxy-lyase were examined. Centrifugation of a cell extract from strain AN256 at 30 000 × *g* for 1 h resulted in only a slight loss of activity from the supernatant and further centrifugation at 150 000 × *g* for 3 h gave a supernatant which still contained half the carboxy-lyase activity of the original cell extract (Table I). These results suggested that a considerable proportion of the enzyme could be separated from the cell membrane by centrifugation and so a partial purification of the enzyme by conventional means was carried out.

Partial purification of the carboxy-lyase

When assaying the carboxy-lyase activities of partially purified preparations, the requirement for any possible co-factors was met by the addition of 0.2 ml of a 30 000 × *g* supernatant from a cell extract of a *ubiD*$^-$, *aroB*$^-$ strain (AN291). This strain was grown in the absence of any precursor of ubiquinone, thus ensuring that the cell extracts lacked not only carboxy-lyase activity (due to the *ubiD*$^-$ allele) but also 3-octaprenyl-4-hydroxybenzoate.

Protamine-sulphate treatment followed by ammonium sulphate precipitation gave approximately a 3-fold purification and a complete recovery of carboxy-lyase activity. The partially purified fraction was then applied to a Sephadex G-100 column, the material eluted with 0.05 M phosphate buffer, pH 7.0, and column fractions assayed for carboxy-lyase activity as described in Fig. 2. One peak of carboxy-lyase activity was found but this was eluted at the void volume with the bulk of the proteins, indicating that the molecular weight was probably in excess of 150 000. Therefore an ammonium sulphate fraction similar to that used above was applied to a Biogel A-5M column and the elution profiles obtained are shown in Fig. 2. There was a single peak of carboxy-lyase activity detected, separated from the bulk of the protein. The specific activity of the carboxy-lyase in the peak fraction was about 24 times the specific activity in the original cell extract. Fractions 53–58 were pooled for further experiments. The recovery of carboxy-lyase activity from the column was about 65 %.

TABLE I

SEDIMENTATION PROPERTIES OF THE CARBOXY-LYASE

Cell extract of strain AN256 was centrifuged at 30 000 × *g* (1 h), and portion of the 30 000 × *g* supernatant centrifuged at 150 000 × *g* (3 h).

Fraction assayed (0.2 ml)	Carboxy-lyase activity (pmol product/h)
Cell extract	2580
30 000 × *g* supernatant	2500
150 000 × *g* supernatant	1290

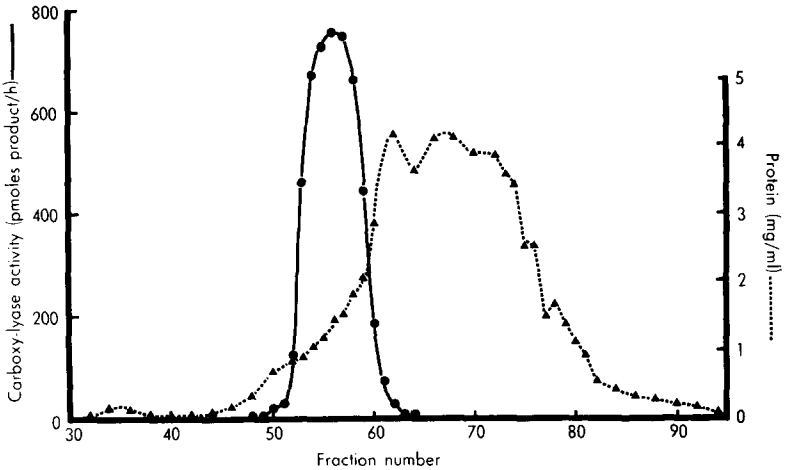


Fig. 2. Bio-Gel A-5M column chromatography of the carboxy-lyase. A 0–50 % ammonium sulphate fraction (15 ml, approx. 40 mg protein/ml, strain AN164) was applied to a 2 × 120 cm column, and 5 ml fractions collected following elution with 0.05 M sodium phosphate buffer, pH 7.0. Fractions (0.2 ml) were assayed for carboxy-lyase activity in the presence of 0.2 ml of a 30 000 × *g* supernatant from strain AN291 (*aroB*$^-$, *ubiD*$^-$), $MnSO_4$ (1 mM), and dithiothreitol (10 mM).

Molecular weight of the carboxy-lyase

The same Biogel A-5M column was calibrated with thyroglobulin (mol. wt., 670 000), human γ-globulin (mol. wt., 160 000) and haemoglobin (mol. wt., 68 000), to obtain an approximate molecular weight for the carboxy-lyase by the method of Andrews [14, 15]. The approximate molecular weight of 3-octaprenyl-4-hydroxybenzoate carboxy-lyase was found to be 340 000.

Requirements for optimal carboxy-lyase activity

As described below, examination of the partially purified carboxy-lyase enzyme after gel filtration showed that for optimum activity the enzyme required a membrane fraction (or phospholipids), a metal ion, dithiothreitol and at least one other so far unidentified soluble co-factor.

The stimulation of carboxy-lyase activity by washed membranes from a cell extract of *E. coli* is shown in Table II. The carboxy-lyase used for these experiments was a protamine sulphate-treated cell extract of strain AN256. It was found that the treatment of cell extract with 90 % of the volume of protamine sulphate solution needed to completely precipitate nucleic acids resulted in a loss of more than 95 % of the total carboxy-lyase activity. The residual activity could be stimulated 5 to 10-fold by the addition of either washed membranes or ethanol-soluble phospholipids (Tables II and III).

Further co-factor requirements for carboxy-lyase activity were shown using enzyme partially purified by gel filtration. In such experiments boiled cell extract of strain AN291 (*ubiD*$^-$) was included as a source of the as yet unidentified low molecular weight co-factor (see below) required for maximum enzymic activity.

The requirement for a metal ion was indicated by the complete inhibition of

TABLE II

STIMULATION OF CARBOXY-LYASE ACTIVITY BY A PARTICULATE FRACTION FROM CELL EXTRACTS

The protamine sulphate precipitation was performed on 30 000 × *g* supernatant of strain AN256 as described in Methods. The supernatants (0.2 ml) were assayed with and without 0.2 ml of a washed membrane preparation prepared from strain AN291 (*ubiD*$^-$, *aroB*$^-$) by centrifugation at 150 000 × *g* for 3 h. The membranes were washed once and resuspended to the original volume in 0.05 M sodium phosphate buffer, pH 7.0. The activities given have been corrected for the dilutions resulting from the addition of protamine sulphate solution.

Protamine sulphate solution added (% of volume needed for complete precipitation of nucleic acids)	Carboxy-lyase activity (pmol product/h) with	
	No addition	Washed membranes
0	6300	6900
35	6800	7600
70	3600	5000
90	270	3300
100	170	2300

TABLE III

STIMULATION OF CARBOXY-LYASE ACTIVITY BY PHOSPHOLIPID

The protamine-sulphate precipitation was performed as described in Methods, using 90 % of the volume of protamine sulphate to give total precipitation of the nucleic acids. The crude phospholipid extract was isolated by a Folch extraction [16] of strain AN164. Ethanol (0.7 ml) was added to dried phospholipid extract from 0.5 g wet wt. cells, briefly heated (37 °C, 5 min) and the ethanol-insoluble material removed. To each tube was added either ethanol (0.05 ml), ethanol (0.05 ml) plus washed membranes (0.2 ml) from strain AN291 prepared as described in Table II, or the ethanolic solution of phospholipid (0.05 ml), followed by the protamine sulphate-treated cell extract of strain AN256 (0.2 ml) and phosphate buffer (see Methods).

Addition to assay	Carboxy-lyase activity (pmol product/h)
None	43
Washed membranes	244
Ethanol-soluble phospholipids	204

carboxy-lyase activity by EDTA. The effect of the EDTA could be overcome by one of a number of divalent metal ions, of which Mn^{2+} was the most effective. In addition it was found that dithiothreitol stimulated the reaction and the effects of EDTA, metals and dithiothreitol are illustrated in Table IV. The stimulation by dithiothreitol was maximal at about 10 mM. Glutathione (10 mM), mercaptoethanol (10 mM), or lipoate (10 mM), gave only slight stimulation of carboxy-lyase activity.

In the absence of boiled cell extract the carboxy-lyase preparation from the Sephadex G-100 column was only slightly stimulated by the addition of the above factors, namely, washed cell membranes, Mn^{2+}, methanol and dithiothreitol. The rate of carboxy-lyase activity was 11 pmol product/h in the absence of any additions,

TABLE IV

EFFECT OF METAL IONS AND DITHIOTHREITOL ON CARBOXY-LYASE ACTIVITY

Each assay tube contained carboxy-lyase preparation (0.2 ml of pooled column fractions from the Biogel A-5M column shown in Fig. 2), supernatant following centrifugation of boiled cell extract (strain AN291, 0.15 ml) and washed membranes (0.05 ml) from strain AN291 (*ubiD*$^-$) prepared as described in Table II (except resuspended to one quarter of the original volume). The EDTA and dithiothreitol were added to give a final concentration of 10 mM, and the metals, 1 mM, in a total volume of 0.55 ml.

Addition	Carboxy-lyase activity (pmol product/h)
None	144
EDTA	2
$MnSO_4$	179
Dithiothreitol	343
Dithiothreitol + $MnSO_4$	611
+ $CoSO_4$	513
+ $CuSO_4$	477
+ $CaCl_2$	415
+ $MgSO_4$	379
+ $ZnSO_4$	296
+ EDTA	3

and 22 pmol product/h when all four factors were included under the usual assay conditions. The addition of supernatant of a cell extract of strain AN291 (*ubiD*$^-$) which had been ultracentrifuged, to the reaction mixture, stimulated the activity to 339 pmol product/h, indicating that at least one other soluble factor was required. This factor was stable to brief boiling and passed through an Amicon PM10 ultrafiltration membrane, indicating that it had a molecular weight of less than 10 000. The ability of the following to replace the soluble factor in the assay were tested, either singly or in various combinations: thiamine pyrophosphate, co-enzyme A, pyridoxamine phosphate, pyridoxal phosphate, biotin, ascorbic acid, NADH, NADPH, folic acid, FAD, FMN, lipoate or glutamate (at 0.2 mM final concentration) or ATP (final concentration 5 mM). Of these compounds, only lipoate stimulated activity slightly (20 %) .Therefore the identity of this compound is unknown at present.

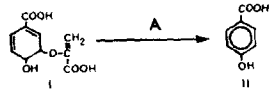
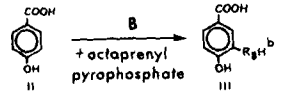
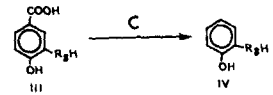
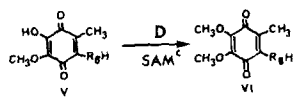
DISCUSSION

One of the interesting aspects of the first *ubiD*$^-$ mutant isolated was that it formed about 20 % of the normal level of ubiquinone although no carboxy-lyase activity could be detected in cell extracts [7]. The present work has confirmed that no carboxy-lyase is detectable in strains carrying this mutant allele and, furthermore, examination of 3 other independently isolated *ubiD*$^-$ strains [17] has shown that each of them forms about 20 % of the normal level of ubiquinone and has no detectable carboxy-lyase activity in vitro. This finding raises the question of a possible alternate metabolic pathway to ubiquinone. However, ubiquinone-deficient strains with complete metabolic blocks have been found in each of the other classes of ubiquinone de-

TABLE V

PROPERTIES OF SOME ENZYMES CONCERNED IN UBIQUINONE SYNTHESIS IN *E. COLI*

The trivial names of the enzymes are (A) chorismate pyruvate-lyase; (B) 4-hydroxybenzoate octaprenyltransferase; (C) 3-octaprenyl-4-hydroxybenzoate carboxy-lyase; (D) 2-octaprenyl-3-methyl-5-hydroxy-6-methoxy-1,4-benzoquinone methyltransferase. The intermediates are (I) chorismic acid; (II) 4-hydroxybenzoic acid; (III) 3-octaprenyl-4-hydroxybenzoic acid; (IV) 2-octaprenylphenol; (V) 2-octaprenyl-3-methyl-5-hydroxy-6-methoxy-1,4-benzoquinone; (VI) ubiquinone. The carboxy-lyase has an additional requirement for an unidentified heat stable factor of low molecular weight. The reactions catalysed by enzymes C and D are stimulated by dithiothreitol.

Reaction catalysed	Approx. mol. wt.	Association with membrane[a]	Metal requirement	Requirement for phospholipid	Reference
	16 000	–	–		[18]
		+	Mg^{2+}		[4, 5]
	340 000	(+)	Mn^{2+}		This paper (see also [5])
	49 000	(+)	Zn^{2+} or Co^{2+}		[6]

[a] + indicates firmly membrane-bound; (+) indicates that the enzyme may be normally associated with the membrane (see text).

[b] R = $-(CH_2CH=\overset{CH_3}{\overset{|}{C}}CH_2-$; [c] SAM, *S*-adenosylmethionine.

ficient mutants described [1] indicating that an alternate pathway is unlikely. Another possible explanation is that there is a second enzyme in whole cells of *E. coli*, capable of decarboxylating 3-octaprenyl-4-hydroxybenzoate, but whose activity is not detected in cell extracts under the conditions used. This would be in agreement with the finding that, on prolonged incubation of a culture of a *ubiD*$^-$ strain, the amount of 3-octaprenyl-4-hydroxybenzoic acid accumulated decreased and there was a corresponding increase in the level of ubiquinone [17]. Since a maximum of 20 % of the normal level of ubiquinone is reached in *ubiD*$^-$ mutants, it is likely that any alternate carboxy-lyase activity has little significance in wildtype strains.

Four of the enzymes concerned in ubiquinone biosynthesis in *E. coli* have now been studied in vitro. Some of their properties are set out in Table V. The substrates for the three enzymes other than chorismate pyruvate-lyase are accumulated in the membranes of the respective ubiquinone-deficient mutants suggesting that they are normally metabolised in the membrane. However, the enzymes catalysing these reactions show varying degrees of association with the membrane. Thus the octaprenyltransferase is firmly associated with the membrane and in cell extracts prepared by the French press, all the activity is associated with the particulate fraction. A considerable portion of both the carboxy-lyase and methyltransferase activities are solubilised by similar treatment but there are other indications that these enzymes normally function in association with the membrane. Thus both enzymes can utilize membrane-bound substrates [17, 6] and the carboxy-lyase is stimulated by the addition of membrane preparations or phospholipid extracts. In studies on the three membrane-associated enzymes using exogenous polyisoprenoid substrates, these have been added in methanol. Methanol has been shown to stimulate carboxy-lyase activity and may do so by increasing the solubility of the substrate, thus facilitating its interaction with the enzyme. It is relevant that in the case of the reaction catalysed by 4-hydroxybenzoate octaprenyltransferase, when the one membrane preparation contains both enzyme and the octaprenyl side chain precursor then methanol has little effect on the reaction rate. If however exogenous solanesyl pyrophosphate is used as substrate, in the absence of membrane-bound side chain precursor, then 17 % methanol is needed for optimum activity [17].

In vitro studies of the enzymes involved in ubiquinone biosynthesis are also in progress with submitochondrial preparations from rat liver [19, 20] and it will be of interest to see how the properties of the individual enzymes in these cells compare with those in *E. coli*.

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